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Subunit stoichiometry of the *Drosophila melanogaster* small nuclear RNA activating protein complex (SNAPc)

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Abstract Small nuclear RNA activating protein complex (SNAPc) is a multi-subunit transcription factor required for expression of small nuclear RNA genes. This protein binds to a promoter element located approximately 40–65 bp upstream of the transcription start site. In *Drosophila melanogaster*, DmSNAPc contains three distinct polypeptide subunits: DmSNAP190, DmSNAP50, and DmSNAP43. The subunit stoichiometry in SNAPc complexed with DNA has not been examined. Therefore, the ability of differently tagged but otherwise identical subunits to associate with each other into the same protein–DNA complex was assayed by antibody super-shift analysis. The results reveal that DmSNAPc contains only a single copy of each of the three subunits.

Structured summary:
MINT-6788068:
DmSNAP43 (uniprotkb:Q9VF25), *DmSNAP50* (uniprotkb:Q7JUY8) and *DmSNAP190* (uniprotkb:Q9VHX0) *physically interact* (MI:0218) by *electrophoretic mobility shift assay* (MI:0413)
MINT-6788151:
DmSNAP190 (uniprotkb:Q9VHX0), *DmSNAP50* (uniprotkb:Q7JUY8) and *DmSNAP43* (uniprotkb:Q9VF25) *physically interact* (MI:0218) by *molecular sieving* (MI:0071)

1. Introduction

The U1, U2, U4, U5, and U6 snRNAs are required for pre-mRNA splicing [1,2]. These snRNAs are synthesized by RNA polymerase II (Pol II), except U6, which is synthesized by RNA polymerase III (Pol III) [3–6]. In animals, a unique promoter sequence termed the proximal sequence element (PSE) is located approximately 40–65 base pairs (bp) upstream of the transcription start site and is essential for basal transcription of snRNA genes by either Pol II or Pol III [3–6]. In insects, this element is termed the PSEA to distinguish it from the PSEB, a less-conserved non-essential promoter element located approximately 25–30 bp upstream of insect Pol II-transcribed snRNA genes [7–9].

The protein that recognizes the PSE (or PSEA) is most commonly called the small nuclear RNA activating protein complex (SNAPc) [10], but it has also been termed PSE binding protein (PBP) [11] and PSE-binding transcription factor (PTF) [12]. In humans, SNAPc/PTF is a complex that contains five distinct subunits known as SNAP190, SNAP50, SNAP45, SNAP43, and SNAP19 [5,6]. The *Drosophila melanogaster* PSEA-binding protein, DmSNAPc (formerly called DmPBP) contains orthologs of only three of these subunits (DmSNAP190, DmSNAP50, and DmSNAP43) [13]. Interestingly, a highly divergent yet homologous complex is required for Pol II transcription of the spliced leader RNA in trypanosomes [14–16]. This suggests that a SNAP-like complex originated very early in eukaryotic evolution.

Despite considerable work on SNAPc, the subunit stoichiometry had not been definitively investigated, particularly when SNAPc is bound to DNA. In the fruit fly, several pieces of data raised the possibility that one or more of the subunits could be present in more than one copy. For example, even though the three subunits individually add up to a molecular mass of only 169 kDa, gel exclusion chromatography indicated that DmSNAPc eluted at a position corresponding to an apparent molecular mass of 375 kDa [17], which would be sufficient for two copies of each subunit. Furthermore, photo-cross-linking data indicate that DmSNAP190 contacts at least 27 bp of DNA [18]. This represents an unusually long region of DNA to be contacted by a single subunit of a DNA-binding protein. Similarly, DmSNAP43 can be cross-linked to a 25 bp stretch of DNA [8,13]. We therefore examined the number of copies of the DmSNAP190, DmSNAP50, and DmSNAP43 subunits in the SNAP complex bound to DNA.

2. Materials and methods

2.1. Source of DmSNAPc

Constructs that code for DmSNAP43, DmSNAP50 and DmSNAP190 in the *Drosophila* expression vector pMT/V5-His-TOPO have been previously described [13]. These include constructs that terminate at the natural stop codon of the protein as well as constructs that have V5 and 6His tags at the C terminus of the protein. Constructs that contain the FLAG-Myc-6His tags were prepared by digesting appropriate constructs with BstBI and MluI to remove the V5 epitope and to replace it with a double-stranded synthetic oligonucleotide coding for the FLAG and Myc epitopes. *Drosophila* S2 cells were co-transfected with a combination of four plasmids (e.g., see Figs. 1A, 2A, and 3A) as well as a plasmid (pCoBlast, Invitrogen) to provide resistance to the antibiotic blasticidin. Following selection, stably transfected cells were induced for 24 h with copper sulfate and lysed in CelLytic M

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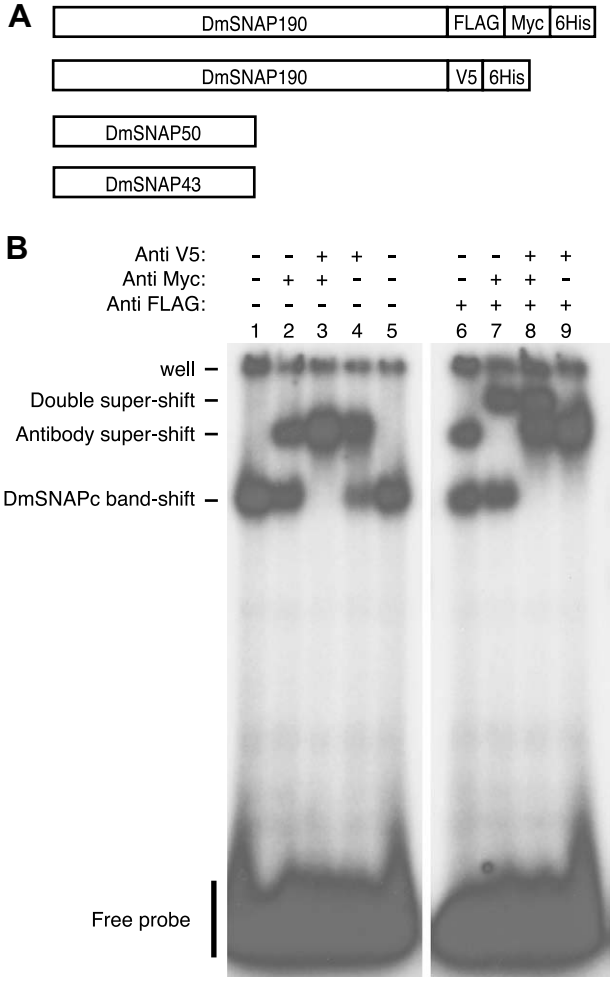

Fig. 1. Stoichiometry of DmSNAP190 in DmSNAPc bound to DNA. (A) Schematic representation of four co-expressed DmSNAPc subunits, including two differently-tagged forms of DmSNAP190. (B) EMSA using nickel column purified DmSNAPc from cells co-expressing the DmSNAP constructs illustrated in part (A). Reactions run in lanes 2–4 and in lanes 6–9 contained added antibodies against the V5, Myc, and/or the FLAG epitopes as indicated above each lane. Lanes 1–5 and 6–9 are from different sections of the same gel.

Reagent (Sigma). The tagged DmSNAPc was then partially purified by nickel column affinity chromatography, dialyzed against BCZ-100 (20 mM HEPES, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 200 μM EDTA, 100 mM KCl, 3 mM DTT, 0.5 mM PMSF, 20% [by volume] glycerol), and concentrated by centrifugation in an Amicon Ultracel 30K centrifugal filtration device to a concentration suitable for use in electrophoretic mobility shift assays (EMSA). Untagged DmSNAPc was purified from wild-type fly embryos as previously described [17].

2.2. DNA probe for EMSA

The radiolabeled probe used for the experiments reported in this paper contained the PSEA sequence of the *D. melanogaster* U1:95Ca gene [previously called the U1 95.1 gene [7]]. It was prepared to have a covalently closed "dumbbell structure" [7,19] to make it resistant to low levels of exonucleases still present in the nickel column fractions. The following two 59-base-long oligonucleotides were each radiolabeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase: 5′-TTGCAATTCCCAACTGGTTTTAGCTGCTCAGCCATGGAAACCTGGCTACTTTCTAGCCA-3′ and 3′-GCTTGGCTTTCCCAAGCAACGTTAAGGGTTGACCAAAATCGACGAGTCGGTACCTTTGG-5′. (The underlined nucleotides indicate the sequences of the PSEA.) They were annealed in equimolar quantities and ligated with T4 DNA ligase; the closed circular dumbbell oligonucleotide was purified by gel electrophoresis prior to use [7].

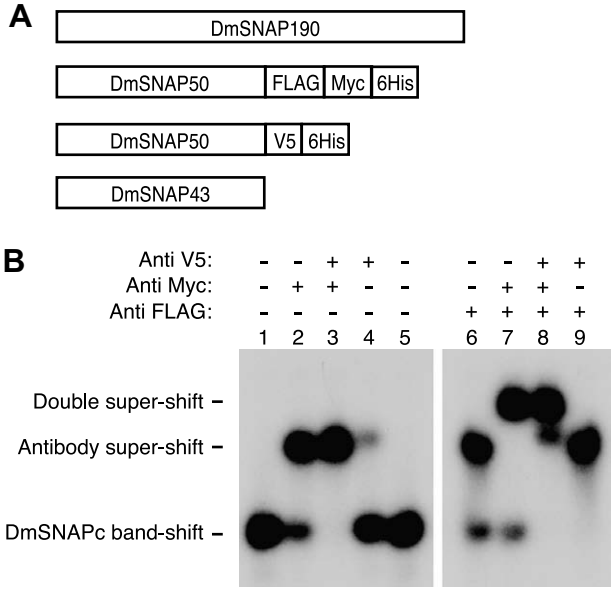

Fig. 2. Stoichiometry of DmSNAP50 in DmSNAPc bound to DNA. (A) Schematic representation of four co-expressed DmSNAPc subunits, including two differently-tagged forms of DmSNAP50. (B) EMSA using nickel column purified DmSNAPc from cells co-expressing the DmSNAP constructs illustrated in part (A).

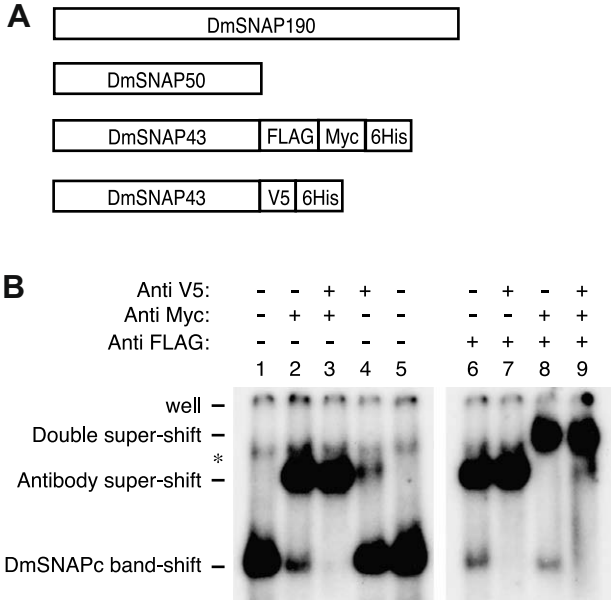

Fig. 3. Stoichiometry of DmSNAP43 in DmSNAPc bound DNA. (A) Schematic representation of four co-expressed DmSNAPc subunits, including two differently-tagged forms of DmSNAP43. (B) EMSA using nickel column purified DmSNAPc from cells co-expressing the DmSNAP constructs illustrated in part (A). The asterisk indicates a non-specific band of unknown origin.

2.3. EMSA conditions

Protein–DNA complexes were formed in a 12 μl reaction volume in BCZ-100. Samples contained 4 μl of protein obtained by nickel column chromatography, 50000 cpm of DNA probe, and 1 μg of poly (dI–dC) • poly (dI–dC). Reaction mixtures were incubated at room temperature for 30 min prior to gel electrophoresis. Monoclonal antibodies (0.7–1.5 μl, when included) were added half-way through the incubation period. Antibodies were the following: anti-V5 (Invitrogen cat.

46-0705); anti-FLAG (Sigma M2 cat. # F1804); and anti-Myc (Sigma cat. # M4439). Samples were electrophoresed in 5% (29:1 acrylamide/bisacrylamide) native gels in a running buffer consisting of 0.025 M Tris, 0.19 M glycine, 1 mM EDTA (pH 8.3).

3. Results

3.1. Stoichiometry of DmSNAP190 in DmSNAPc bound to DNA

To investigate the stoichiometry of the DmSNAP190 subunit, S2 cells were simultaneously co-transfected with the four constructs shown in Fig. 1A. The cells co-expressed two differently tagged forms of DmSNAP190. One form had tandem FLAG and Myc tags preceding the 6His tag, whereas the other form contained a V5 tag preceding the 6His tag (Fig. 1A). The tagged DmSNAPc thus obtained was employed for EMSA and super-shift analysis with a DNA fragment that contained the PSEA of the *D. melanogaster* U1:95Ca gene.

Fig. 1B lanes 1 and 5 show the position of the shifted band that results from tagged DmSNAPc alone binding to the DNA fragment. Inclusion of either Myc antibody (lane 2) or V5 antibody (lane 4) resulted in the appearance of a super-shifted band, but in both cases a portion of the labeled fragment remained at the original band-shift position. Addition of greater amounts of either antibody alone did not significantly increase the amounts of super-shifted complex (data not shown). Thus the antibodies were present in saturating amounts. On the other hand, when both Myc and V5 antibodies were added to the same reaction, all of the protein–DNA complex was super-shifted and appeared at the same position (lane 3). Thus, as expected, all the DmSNAPc activity in the nickel column fraction contained either a Myc or V5 tag.

These results represent the pattern expected if there is only one copy of DmSNAP190 (that has either a Myc tag or a V5 tag) in the DmSNAPc–DNA complex. The simplicity of the observed pattern is not easily reconciled with there being two or more DmSNAP190 subunits present in the complex. For example, if there were two DmSNAP190 subunits in DmSNAPc, it becomes necessary to hypothesize that the band labeled ''antibody super-shift'' is due to the binding of two antibodies to the complex. But if there were two copies of DmSNAP190, ''heterodimers'' of Myc- and V5-tagged DmSNAP190 should exist in some of the DmSNAPc–DNA complexes, and in lanes 2 and 4 these would be expected to migrate to a position intermediate between the two observed bands. No evidence appears for the existence of such intermediate complexes.

Lanes 6–9 in Fig. 1B show results from similar assays but in addition including antibodies against the FLAG epitope. The FLAG epitope is present on the same polypeptide as the Myc epitope (Fig. 1A). As expected, FLAG antibody alone gave the same pattern as the Myc antibody alone (compare lanes 6 and 2). Likewise, the FLAG antibody and the V5 antibody together gave the same result as the Myc and V5 antibodies together (compare lanes 9 and 3). However, the FLAG antibody and the Myc antibody when added together (lane 7) caused a further super-shift resulting from the binding of both FLAG antibody and Myc antibody to FLAG/Myc-tagged DmSNAP190 present in the DmSNAPc–DNA complex. This clearly demonstrates that more than one antibody can bind to the complex to provide a double super-shift. Further addition of the V5 antibody (lane 8) super-shifted the lower band observed in lane 7 (which must contain only V5-tagged DmSNAP190); in contrast, the V5 antibody had no effect on the double super-shifted band that resulted from the binding of the FLAG and Myc antibodies. These experiments provide strong evidence that there is only one DmSNAP190 subunit in the protein–DNA complex.

3.2. Stoichiometry of DmSNAP50 in DmSNAPc bound to DNA

To investigate whether DmSNAP50 is present in one or more than one copy in DmSNAPc, the four constructs shown in Fig. 2A were co-expressed in stably-transfected *Drosophila* S2 cells and used for EMSA (Fig. 2B). In this figure, only the upper part of the gel that pictures the protein-retarded bands is shown.

Addition of Myc antibody by itself super-shifted the majority, but not all, of the DmSNAPc–DNA complex (compare lane 2 with lane 1). Inclusion of V5 antibody alone super-shifted a reciprocal minority of the complex (lane 4). This result indicates this cell line was expressing more of the FLAG-Myc-tagged DmSNAP50 than of the V5-tagged DmSNAP50. When both Myc and V5 antibodies were included in the incubation prior to loading onto the gel, the entire signal from the DmSNAPc–DNA complex was super-shifted (lane 3). These results parallel those obtained with the tagged DmSNAP190 protein (Fig. 1B). Due to the expression ratio of the differently tagged forms of DmSNAP50, most of the V5-tagged subunits should exist in ''hetero'' complexes with Myc-tagged DmSNAP50 if there were more than one copy of DmSNAP50 in DmSNAPc bound to DNA. However, Fig. 2 provides no evidence for the existence of such ''hetero'' complexes with intermediate mobilities.

Addition of FLAG antibody to the reaction (Fig. 2B, lanes 6–9) further retarded the mobility of protein–DNA complexes that contained the Myc epitope (upper bands in lanes 7 and 8) but had no effect on complexes that contained the V5 epitope (lower bands in lanes 7 and 8). Together, these results indicate that DmSNAP50, like DmSNAP190, is present in only one copy in DmSNAPc complexed with DNA.

3.3. Stoichiometry of DmSNAP43 in DmSNAPc bound to DNA

Finally, to investigate the stoichiometry of DmSNAP43 in the DmSNAP complex, similar experiments were performed with DmSNAPc containing differentially tagged DmSNAP43 (Fig. 3). The pattern of band-shifts, super-shifts, and double super-shifts with tagged DmSNAP43 was essentially the same as seen above when tagged DmSNAP190 or tagged DmSNAP50 was used. (As noted above the lanes, the arrangement of lanes 6–9 with respect to antibody addition is different in Fig. 3B relative to Figs. 1B and 2B.) In these cells, the FLAG-Myc-tagged version of the tagged protein was again expressed at a higher level than the V5-tagged version, but bands arising from DmSNAPc carrying the V5-tagged version of DmSNAP43 were clearly visible. From these results, we conclude that there is only a single copy of DmSNAP43 in DmSNAPc bound to DNA.

3.4. The epitope tags do not alter the subunit stoichiometry of DmSNAPc

We also investigated the possibility that the presence of the tags might artifactually affect the stoichiometry of DmSNAPc by interfering with the multimerization properties of the

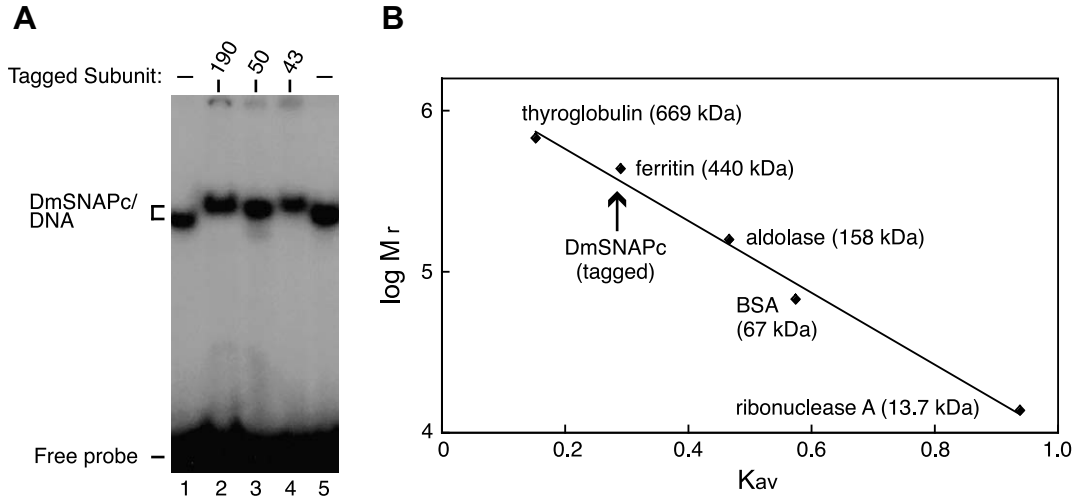

Fig. 4. Chromatographic properties of DmSNAPc are not significantly affected by the presence of the C-terminal epitope tags. (A) EMSA of tagged and untagged DmSNAPc. Reactions shown in lanes 1 and 5 contained untagged DmSNAPc from fly embryos [17]. Reactions run in lanes 2–4 contained DmSNAPc tagged on the subunit indicated above the respective lanes. The amount of each protein sample was adjusted to give shifted bands of similar intensities. (B) Gel exclusion chromatography of DmSNAPc tagged on the DmSNAP43 subunit. Sephacryl S-300 HR chromatography was carried out exactly as previously described [17] and DmSNAPc activity was determined by EMSA of the elution fractions following approximately 15-fold concentration.

subunits. We first performed EMSAs in which the tagged complexes were run side-by-side with untagged DmSNAPc purified from wild-type fly embryos [17]. Fig. 4A shows that the three tagged DmSNAPc–DNA complexes each ran with a relative mobility just slightly less than the untagged DmSNAPc–DNA complex. The slower mobility is consistent with the additional mass provided by the tags and at the same time strongly suggests that normal SNAP subunit interactions are maintained in the tagged complex.

We also examined the properties of tagged DmSNAPc by gel exclusion chromatography (Fig. 4B). DmSNAPc tagged on DmSNAP43 eluted with nearly the same apparent molecular mass (372 kDa) previously reported for untagged DmSNAPc [17]. Together, these experiments provide strong evidence that the tags do not affect the normal subunit composition of DmSNAPc.

4. Discussion

4.1. Only one copy of each DmSNAP subunit exists in the DmSNAPc–DNA complex

Prior to this work the stoichiometry of the subunits of SNAPc from *Drosophila* or other metazoans had not been systematically investigated. We previously found that DmSNAPc eluted from a gel exclusion column with a molecular mass of ~375 kDa relative to globular protein standards [17]; this size is sufficient to accommodate two copies of each subunit. Human SNAPc/PTF possesses an apparent molecular mass of ~500 kDa by gel exclusion chromatography [12] but ~200 kDa by glycerol gradient sedimentation [10,12]. The latter is clearly an underestimate since there are five different subunits in human SNAPc that individually have a combined molecular mass of ~343 kDa [20]. In the trypanosome, *Leptomonas seymouri*, the three cloned subunits of SNAPc have a combined molecular mass of 139 kDa [14] and, by combining the results of gel exclusion chromatography and glycerol gradient centrifugation, an estimated molecular mass of 122 kDa [21]. These data suggest that there is one copy of each subunit in trypanosomal SNAPc free in solution.

However, a more important question, the configuration of DmSNAPc bound to DNA, had never been examined. For example, DmSNAPc potentially could bind to DNA as a "dimer of heterotrimers". The data presented herein rule out that possibility and provide direct evidence that there is only one copy of each DmSNAP subunit in the protein–DNA complex formed on the PSEA.

4.2. Metazoan SNAPc as an elongated asymmetrical complex

It appears that metazoan SNAPc is a highly asymmetrical and elongated complex. This is consistent with the fact that gel exclusion chromatography overestimates the molecular mass of SNAPc [12,17] and glycerol gradient sedimentation underestimates its mass [10,12]. Moreover, DmSNAPc is in close contact with at least 40 bp of DNA when it binds to a U1 gene PSEA [8,13]. If DmSNAPc were a spherical molecule, it would have to significantly wrap or bend the DNA to remain in contact with four turns of the DNA helix. However, DmSNAPc only modestly bends the PSEA based upon circular permutation, minicircle binding, and ligase-catalyzed circularization assays [22]. Moreover, human SNAPc/PBP was found not to bend DNA [11]. Thus metazoan SNAPc is very likely a highly asymmetrical protein complex that is significantly elongated along the axis of it contacts with the DNA.

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